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Targeting Epithelial Cell Migration To Accelerate Wound Healing

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14. ABSTRACT

Successful wound healing requires the recruitment and migration of distinct cell types to the wound followed by re-epithelialization of the surface of injured tissue. Interventions that enhance the migration of effector cells to the site and temporally increase epithelialization can be clinically relevant. Cell attachment and adhesion molecules necessary for cell migration include all four members of the RIPP complex, consisting of the proteins **R**su1, **I**ntegrin Linked Kinase (ILK), **P**INCH, and **P**arvin. The correct association of these proteins in a functional complex depends on their phosphorylation by serine threonine kinases of the protein kinase C (PKC) family and the process can be enhanced or inhibited by modulating the levels of the RIPP complex proteins as well by regulating their serine and threonine phosphorylation. Our data indicate that the expression of the RIPP proteins is required for migration of human keratinocyte cell line *in vitro*. In addition, the phosphorylation of RIPP proteins contributes to their association and complex formation. For Rsu1- the sites include serine 264 and 268. Formation of the RIPP complex depends on appropriate signals that derive from cell adhesion including from PKC pathway and, hence, the inhibition of PKC blocks Rsu1-PINCH1 association, PINCH1-ILK association, and cell migration. Characterization of migration in HACAT cells indicates that PKCδ may modulate migration on two-dimensional surface.

15. SUBJECT TERMS
Wound healing, cell migration, protein kinase C

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Introduction

The ultimate goal of this work is directed toward improved treatment protocols to accelerate wound healing. The rationale is that clinical interventions that enhance the migration of effector cells to wound sites may hasten epithelialization and are relevant for wound healing. Successful wound healing requires the recruitment and migration of distinct cell types to the wound followed by re-epithelialization of the surface of injured tissue. Interventions that either enhance the migration of effector cells to the site or temporally increase epithelialization are potentially clinically relevant. This study will determine if a complex of proteins required for epithelial cell migration can be manipulated in tissue culture and a mouse wound model to enhance re-epithelialization. The animal studies will employ a gel encapsulated release system in wounds to deliver biomodulators for regulation of specific protein expression in migrating epithelial cells.

The cell attachment and adhesion molecules necessary for this cell migration that are the focus of the study includes members of the RIPP complex, an intracellular adhesion complex consisting of the proteins Rsu1, Integrin Linked Kinase (ILK), PINCH, and Parvin (2,3). The RIPP complex serves a major intracellular effector complex for integrin-mediated adhesion and migration. ILK binds to the cytoplasmic domain of the beta integrins via its carboxyl terminal domain; the other members of the complex are found in a complex with ILK. The complex is linked to the epidermal growth factor receptor (EGF-R) signaling by members of the Nck family of signaling proteins. This is significant because EGF is a major chemo-regulator of epithelial cell migration in wound healing. In addition, the correct association of these proteins in a functional complex depends on their phosphorylation by serine threonine kinases including those of the protein kinase C (PKC) family, suggesting that they may serve as "druggable" targets affecting the re-epithelialization of wounds(4). The PKCη isoform has been particularly singled out as a modulator of epithelial cell migration implicating it as the potentially relevant PKC isoform. The above cited study also demonstrated that the depletion of PKCη directly altered epithelial cell migration *in vitro* (1).

The above studies have led to the formulation of the following hypothesis: Epithelial cell migration into wounds can be temporally regulated by the association of Rsu1-ILK-PINCH1-Parvin into a functional complex. This process can be enhanced or inhibited by modulating the levels of the proteins that constitute the RIPP complex as well by regulating their serine and threonine phosphorylation. The topical administration of pharmacological regulators of serine and threonine protein kinase(s) may influence RIPP complex formation locally in wounds.

Body the results are those obtained in year 1

Task 1. Determination of the RIPP protein phosphorylation profile in HaCAT cells following exposure to chemical inducers of PKCη. The sites of phosphorylation on PINCH1 and Rsu1 proteins with and without PKC activation will be identified by ^{32}P labeling of HaCAT cells and, if feasible, primary human keratinocyte cultures cells. Cells will be treated with specific activator of PKC (phorbol ester) and/or inhibitors of PKC (BIM, calphostin). In addition, cells will be labeled for recovery of PINCH1 and Rsu1 following siRNA-mediated depletion of individual PKC homologs; this approach will detect contributions of all PKC proteins to the phosphorylation events. PINCH1 and Rsu1 will be recovered using specific antibodies for the respective proteins; the detection of phosphorylation and quantitation of phosphate addition will be performed by SDS-PAGE, transfer to PVDF membrane, autoradiography and β-scanning of membrane. The amount of PINCH1 and Rsu1 in the immunoprecipitates will be determined by western blot of the filters. This will establish the specific PKC proteins and their activation pathways leading to phosphorylation of PINCH1 and Rsu1 as well as cell stimulation conditions for Task 2. Months 1-3.

Rsu-1 is phosphorylated at consensus PKC sites. Analysis of the predicted amino acid sequence of Rsu-1 identified consensus sites for phosphorylation by PKC, PKA and casein kinase II. The phosphorylation of PINCH1 and Rsu1 proteins with and without PKC activation was performed by ^{32}P

labeling of cells and stimulation with specific activator of PKC (phorbol ester) in presence or absence of inhibitors of PKC (BIM, calphostin). PINCH1 and Rsu1 were recovered using specific antibodies for the respective proteins or the engineered tags; the detection of phosphorylation and was performed by SDS-PAGE, transfer to PVDF membrane, autoradiography and β-scanning of membrane. The amount of PINCH1 and Rsu1 in the immunoprecipitates was determined by western blot of the filters. Preliminary experiments demonstrated that Rsu-1 is phosphorylated on serine in response to stimulation of cells with growth factor or TPA. The HA-tagged mutant Rsu-1 proteins were expressed following plasmid transfection into cells, and the incorporation of ^{32}P into Rsu1 following TPA stimulation of the cells was determined. The experiment in figure 1 demonstrates that pretreatment of cells with the PKC inhibitors, BIM or long term TPA, blocked HA-Rsu-1 phosphorylation but that tyrphostin did not block phosphorylation. Additional inhibitors that did not block phosphorylation in response to TPA include inhibitors targeting Mek-Erk (PD98059), PI-3-kinase (Wortmannin), and casein kinase II (DRB). These data suggest that the sites for Rsu-1 phosphorylation are near the N terminus and COOH terminus of the molecule and that activation of PKC (and/or kinases downstream of PKC) can contribute to Rsu1 phosphorylation.

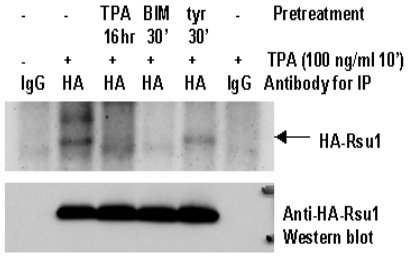


Figure 1. HA-tagged Rsu-1 was transfected into cells and at 48 hours post tranfection the cells were labeled with ^{32}P orthophosphate for 4 hours. Prior to stimulation the cells were treated with TPA (phorbal 12-myristate 13-acetate), (400ng/ml) for 16 hr or BIM (bisindole maleimide 20 nM) or tyr (tyrphostin-A25 10 μM) for 30 minutes. The cells were stimulated with TPA (100ng/ml) for 10 minutes, lysed and the HA-Rsu-1 was recovered by immuno-precipitation with anti HA antibody. The immunoprecipitates were separated by SDS-PAGE and transferred to filters for direct autoradiography and western blot with anti HA antibody.

Determination of the effect of siRNA-mediated depletion of individual PKC homologs on PINCH1 and Rsu1 phosphorylation and binding are on-going. These analyses will detect contributions of individual PKC proteins to the phosphorylation events.
Site directed mutagenesis converting Rsu1 serine residues to alanine was performed on 8 potential PKC or casein kinase II phosphorylation sites (S4, S23, T135, S163, T241, S264,S268) (Appendix item-1). The phospho-mutant Rsu1 proteins were expressed in cells and following TPA stimulation were recovered and examined for phosphorylation. The results indicated that Rsu-1 serine 4 and serine264/serine268 mutants were significantly less phosphorylated than mutants at S23, S163, T135 and T241 in this assay.

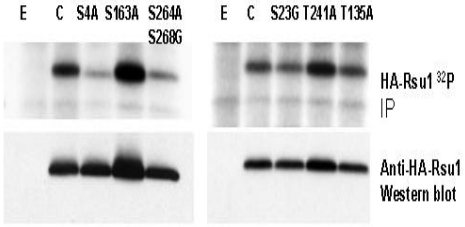


Figure 2. HA-Rsu-1 and the HA-tagged Rsu-1 mutants were transiently transfected into cells which were labeled as described above. E= empty vector, C= wild type HA-Rsu1. Mutants are as indicated in figure labels.

The association of Rsu-1 and PINCH1 requires signal transduction, possibly from Protein Kinase C.
Because Rsu1 and PINCH1 co-precipitated in mammalian cells, studies were undertaken to examine the effects of cell physiology and signaling on the association of the proteins. Cell starvation by serum removal for 16 hours resulted in an inhibition of co-precipitation even though the synthesis of the proteins did not decline. Further experiments, revealed that stimulation of co-transfected cells with serum or phorbol-myristate (TPA) resulted in re-association of the HA-Rsu-1 and myc-PINCH1 proteins.

These results suggested that protein kinase C stimulation and Rsu1 phosphorylation enhanced the binding of HA-Rsu-1 and myc-PINCH1.

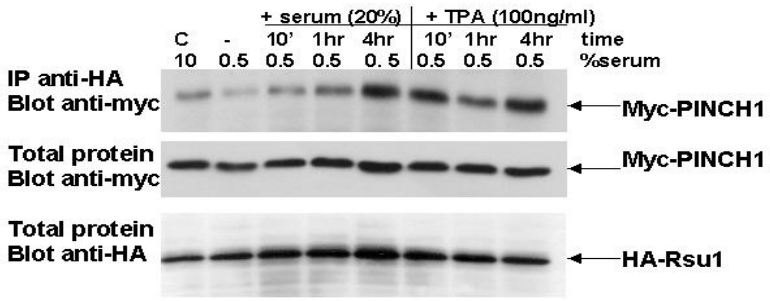


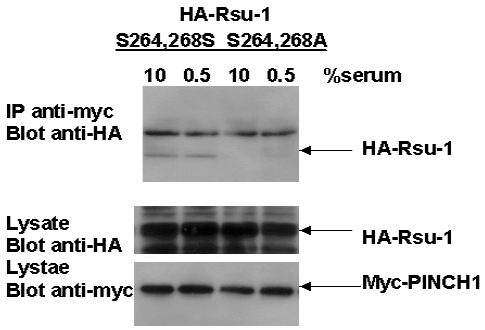
Figure 4. Cells transfected with a HA-Rsu1 and myc-PINCH1 plasmids. 36 hours post transfection the serum concentration was reduced to 0.5% except in the control well. Following 16 hours of serum starvation the cells were stimulated with either 20% serum or TPA. Cell lysates were harvested at 10min, 1 hr and 4 hours post stimulation. The HA-Rsu1 protein was immunoprecipitated with anti HA antibody. The immunoprecipitates (top panel) were analyzed by western blotting with anti myc Ab to detect associated myc-PINCH1 protein. The second and third panels show the amount of myc-PINCH1 or HA-Rsu-1 in 10% of each lysate

Additional studies have demonstrated that mechanical detachment of cells from substrate also blocks the association of Rsu1 and PINCH1. Detachment also inhibits numerous signal transduction pathways, suggesting that the detachment-induced block of Rsu1-PINCH1 binding may also result from decreased phosphorylation.

Task 2: The sites of phosphorylation on RIPP proteins PINCH1 and Rsu1 with and without PKC activation are being identified by mass spectrometry following recovery using specific antibodies. Initial attempts were not successful and the analysis had to be repeated but current data identify S264 and S268 as phosphorylation sites on Rsu1.

Task 3: Existing PINCH1 and Rsu1 phosphorylation site mutants will be tested, and some additional mutants constructed, for in vitro phosphorylation following activation of PKC pathway. This will confirm identities of the specific sites of phosphorylation. Month 6-9.

Figure 5. Cells were co-transfected with a myc-PINCH1 plasmid + HA-Rsu1 plasmid, or HA-Rsu1 (S264,268A) plasmid. 36 hours post transfection the cells were incubated in 10% or 0.5% serum concentration Following 16 hours of serum starvation cells were harvested. The myc-PINCH1 protein was immunoprecipitated with anti myc antibody and subjected to SDS-PAGE and western blotting with anti HA Ab to detect associated HA-Rsu1 protein. The bottom panel shows the amount of myc-PINCH1 and HA-Rsu1 or mutant HA-Rsu1 in each lysate prior to immunoprecipitation.



Plasmids encoding the mutant HA-Rsu-1 proteins S4A and S264A/S268A were tested for the ability to co-immunoprecipitate with myc-PINCH in Cos1 cells. The results shown in Figure 4 indicated that mutation of S264 and S268 blocked the co-precipitation with myc-PINCH1 suggesting that phosphorylation at these sites is required for the association in mammalian cells. Mutation of S4 did not affect co-immunoprecipitation with PINCH1. A recent report indicated that PKC activity is also required for PINCH1 and ILK association.

Task 4. The effect of PKC activation and RIPP phosphorylation on HaCAT cell and primary human keratinocyte migration. The effect of PKC activation and RIPP phosphorylation on HaCAT cell and primary human keratinocyte migration on extracellular matrix substrates will be analyzed. Cells will be seeded in microtiter plates and monolayers will be “wounded/scarred” and then migration into scarred area will be monitored by quantitative microscopic (light and fluorescent) imaging. The parameters

tested will include treatment of cells for PKCη activation and inhibition as well as siRNA-mediated depletion of specific PKC proteins. Months 1-4.
Depletion of Rsu1 protein inhibits cell migration in vitro. Cells were transfected using siRNA for Rsu1, PINCH1, ILK or negative control siRNA. The cells were plated in Oris^R migration plates containing an insert that was removed at 36 hours past migration to allow documentation and quantification of cell migration. The results (Figure 6) indicated that the depletion of PINCH1 or ILK inhibited cell migration in response to EGF. Rsu1 siRNA (Rsu1-3) that depletes all the Rsu1 variants (i.e.p33,p29,p27,p15) blocked migration but Rsu1 siRNA that depletes only full length p33 Rsu-1 does not block migration compared to the negative control siRNA. This identifies a role for Rsu1 alternative splice products in regulating migration *in vitro*. Note that depletion of Rsu1 results in reduction in Rsu1 and PINCH1, and that PINCH1 depletion reduces Rsu1 and ILK as well as PINCH1.

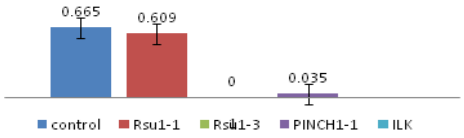
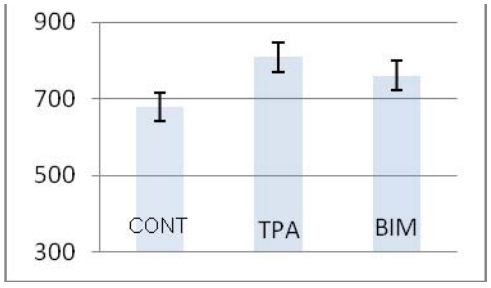


Figure 6. Cells were plated in Oris^R migration plates containing an insert that was removed at 36 hours past migration to allow documentation and quantification of cell migration. Cells were photographed at the beginning of the migration assay and at the conclusion 20 hours later. Quantitation was performed by staining cells and reading absorbance spectrophotometrically using a template to define the field of migration. The results are reported as the mean of four wells for each condition. Error bars represent standard error. T test result: Control:Rsu1-3. PINCH1-1 or ILK siRNAs p<0.005

The effect of enhancement or inhibition of PKC signaling on human keratinocyte migration has been tested *in vitro*. HaCAT cells treated with TPA or inhibitors of PKC (BIM and calphostin C) were plated on Oris^R cell migration plates and the degree to which the cells migrated into the empty surface was recorded after 24 hours.

Distance not filled during 24 hr HaCAT migration



Migration of HaCAT Cells (% closure)

Untreated control	**24.4%**
TPA (10 ng/ml)	**10.1%**
BIM (20nM)	**15.5%**

Figure 7. TPA inhibits HaCAT cell migration. HaCAT cells harvested, exposed to media alone or inhibitor + media and plated in Oris^R migration plates containing a gel insert that dissolved within approximately 2 hours after cells were added to the wells. The insert prevented attachment of cells to a circular area in the center of each well; migration is required for the cells to fill the center. At 24 hours post plating the results of the migration were examined. The cells were fixed, stained and photographed to allow documentation and quantification of cell migration. Quantitation was performed by photographing microscopic images and applying measurement software to calculate distance remaining in the center of the well. The results are reported as the mean of four wells for each condition. Error bars represent standard error. T test: CONT:TPA, p<0.005; CONT:BIM, p<0.05. TPA (phorbal 12-myristate 13-acetate), BIM (bisindole maleimide 20 nM).

Additional studies in figure 8 demonstrated that inhibition of Mek-Erk, and JNK pathways decreased HaCAT migration. Among the PKC inhibitors Rotterlin resulted in the most significant inhibition of migration, suggesting a role for PKCδ. As shown above the addition of BIM had little effect. Inhibition of pathways known to contribute to migration via integrin and actin-dependent functions, Rac, Rho Kinase, Rap1A, and PKA, also inhibited migration. PI-3-kinase inhibitors and rapamycin, the inhibitor of mTOR, had limited or no effect on migration of HACAT cells.

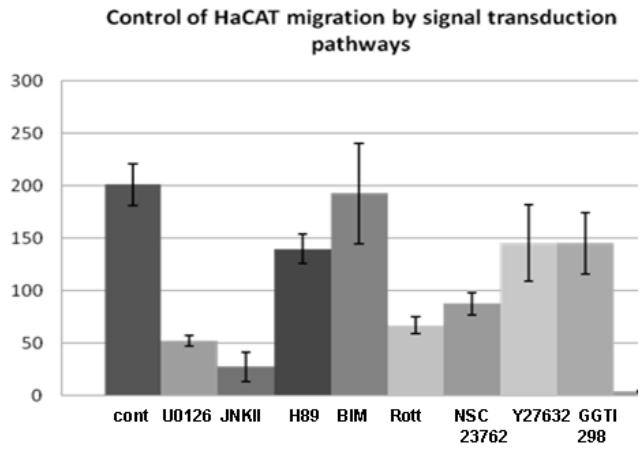


Figure 8. HaCAT cells plated in Oris^{R} migration plates containing a removable insert. The insert prevented attachment of cells to a circular area in the center of each well; migration is required for the cells to fill the center. At 24 hours post plating the inserts were removed and inhibitors were added to the media. The results of the migration were examined 36 hrs later. The cells were fixed, stained and quantification of cell migration was performed by spectroscopy. The results are reported as the mean of four wells for each condition. Error bars represent standard deviation. Inhibitor target and concentrations were as follows: 20 μM U0126 (Mek), 1 μM JNKII inhibitor (JNK), 100nM H89 (PKA), 20nM BIM (bisindole maleimide) PKC, 4 μM Rotterlin (PKCδ), 25 μM NSC23762 (Rac), 200 nM Y23632 (Rho kinase), 5 μM GGTI298 (RAP1A). T test results: CONT:U0126,JNKII, H89, Rotterlin, NSC23672 $p<0.005$; CONT:Y27632 and GGTI298 $p<0.05$. CONT:BIM, not significant.

Task 5. HaCAT cell will be infected with viral vectors encoding wild type and phosphorylation site and phospho-mimetic mutants of PINCH1 and Rsu1 following siRNA-mediated knockdown of the respective endogenous protein. The cells will be used to test the effect of the mutants on *in vitro* migration of cells as described above. The results will determine the role of PINCH1 and Rsu1 phosphorylation on the effectiveness of the complex to contribute to migration and will identify the mutants of PINCH1 and Rsu1 to be used in *in vivo* wound healing studies. Months 5-9

The lentiviral vectors have been constructed and the replication defective virus has been purified. We determined that virus purification is necessary because components of the 293T produced cell conditioned media enhanced migration non-specifically. HaCAT cell infected with viral vectors encoding wild type and phosphorylation site and phospho-mimetic mutants of PINCH1 and Rsu1 following siRNA-mediated knockdown of the respective endogenous proteins are being tested by this method. The preliminary data indicates that the Rsu1 phosphorylation site mutants fail to replace wildtype Rsu1 in migration assays. The results will determine the role of PINCH1 and Rsu1 phosphorylation on the effectiveness of the complex to contribute to migration *in vitro.*

Experiments to identify the individual PKC isoforms that contribute to migration are ongoing. Cells are seeded in microtiter plates as above and following removal of the central plug the migration into central area of the wells migration is monitored by quantitative microscopic (light and fluorescent) imaging. We have included the use of fluorescent dye addition to cells just prior to analysis to improve quantitation of the migration analysis. The parameters tested include treatment of cells for PKCη activation and inhibition as well as siRNA-mediated depletion of specific PKC proteins. Studies to examine the effect of depletion of PKCδ on migration are complicated by effects of PKCδ depletion on cell cycle progression in non-transformed HaCAT or keratinocyte cultures. The experiments are ongoing and will be completed within the next month.

Task 6. Modulating the specific levels and the phosphorylation of RIPP complex proteins in an *in vivo* model of wound healing. Mouse punch wounds will be exposed to 1) gel-encased siRNA (or morpholinos) to deplete or 2)viral vectors to elevate wildtype and phosphomimetic mutants of the PINCH1 and Rsu1 proteins. The effect on temporal wound closure will be assessed by image analysis of wound. Infection will be verified by GFP fluorescence and immune-staining for specific vector-expressed or siRNA-depleted proteins. Immunohistochemistry with specific stains and immunofluorescent staining will be used to determine the effect on individual cell types in wounds. Detection and analysis of granulation tissue, collagen, fibronectin, metalloproteinase MMP9 will be performed.

The studies on the effect of modulating PKCη and RIPP protein in vivo are underway. Because of the cost of the animal experiments and the specific number of animals approved, it is essential to have completed the biochemical and in vitro analysis before performing the *in vivo* studies.
We have requested a no cost extension in order to complete the *in vivo* experiments.

Key Accomplishments:
-Rsu-1 is phosphorylated in response to activation of PKC at consensus PKC sites.
-The association of Rsu-1 and PINCH1 requires phosphorylation at Protein Kinase C sites.
-Inhibiton of cell adhesion blocks Rsu1 and PINCH1 association.
-Depletion of Rsu1, PINCH1 or ILK inhibits migration *in vitro*.
-Disruption of PKCδ signaling inhibits migration of HaCAT cells.

Reportable Outcomes:
- *"Mammary Epithelial Cell Changes in Adhesion and Migration by Alteration of RSU1 Expression"* Reyda Gonzalez-Nieves, John Buckingham, ML Cutler. Abstract Annual Meeting, American Association for Cell Biology Meeting, December 2009, San Diego, CA.

Conclusions: The expression of the RIPP proteins is required for migration of human keratinocyte cell line *in vitro*. In addition, the phosphorylation of RIPP proteins contributes to their association and complex formation. For Rsu1- the sites include serine 264 and 268. Formation of the RIPP complex depends on appropriate signals including those that derive from cell adhesion. These signals appear to include those from PKC pathway and, hence, the inhibition of PKC blocks Rsu1-PINCH1 association, PINCH1-ILK association, and cell migration. The completion of the remaining *in vitro* studies and the determination of the effect of PKC modulation on *in vivo* migration will enhance understanding of regulation of migration by RIPP proteins. The potential for this information to enhance understanding of the signaling requirements for *in vivo* migration is significant.

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APPENDIX

Item 1.
Primers used for site-directed mutagenesis of Rsu1 consensus phosphorylation sites:

S4A: 5' CAGATTACGCTGGTTCCAAGGCACTGAAGAAGCTGGTG
3' CACCAGCTTCTTCAGTGCCTTGGAACCAGCGTAATCTG
S23G: 5' GGAAGTGGACATGGGTGACAGGGGTATCTCC
3' GGAGATACCCCTGTCACCCATGTCCACTTCC
T135A: 5' GGAAACTTCTTCTACCTCACCGCCCTGGCACTCTATCTAAGC
3' GCTTAGATAGAGTGCACGCAGGGCGGTGAGGTAGAAGAAGTTTCC.
S 163G: 5' GTTGCAGATACTCGGCCTCAGGGATAATGACC
3' GGTCATTATCCCTGAGGCCGAGTATCTGCAAC
T241A: 5' GCTTACAAGTACCTCTACGGCAGACACATGCAAGCGAAC
3' GTGTCTGCCGTAGAGGTACTTGTAAGCTTCTGAACGAA
S264A / S268G:
5' AAACCAAAAAGATCGGCCGGAAACCCCTAGCA
3' CGGCCGATCTTTTTTGGTTTGTCGTTATTCTTCTTTGG